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Award Number: DAMD17-03-1-0329

TITLE: Repairing RNA Transcripts that Mediate Breast Cancer Susceptibility

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REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE
Repairing RNA Transcripts that Mediate Breast Cancer Susceptibility

5. FUNDING NUMBERS
DAMD17-03-1-0329

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

A number of genetic mutations that predispose individuals to cancer are known. For example, over 25 insertion mutations have been identified in BRCA1 and p53 transcripts that have been linked to breast cancer susceptibility. Each mutation impairs the functionality of the resultant tumor suppressor protein. Developing technology to specifically excise these mutations, thus restoring tumor suppressor activity, would be of considerable importance for the development of new molecular-based therapeutics. To this end, we have developed a novel biomolecule (a ribozyme) that can specifically excise regions from RNA transcripts. In this work, we designed a ribozyme that excises an insertion mutation that is linked to breast cancer predisposition from a short mimic of the p53 transcript in a cell-free system. Furthermore, we developed a Green Fluorescent Protein system to demonstrate that such ribozymes can excise designated insertion mutations from transcripts within bacterial cells. This ribozyme is not toxic to the cell, and appears to work with a reasonable degree of specificity. The successes in this study represent the foundation for a completely novel, yet simple molecular-based therapeutic strategy: repair disease-causing RNA transcripts by removing their insertion mutations, thereby restoring the proper function of the resultant biological products.

14. SUBJECT TERMS
Drug development, tumor suppressor genes, development and character of model systems, complementary and alternative treatment approaches

15. NUMBER OF PAGES
31

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The development of technologies for identifying genetic mutations that lead to diseases is rapidly outpacing the development of therapeutic strategies to remediate the effects of these mutations. This work directly addresses this discrepancy by analyzing a new potential therapeutic strategy. To this end, we are analyzing the potential of a novel catalytic RNA, which we previously developed, to excise a single base insertion mutation from a transcript derived from the p53 gene. This mutation impairs the functionality of the resultant tumor suppressor protein product, leading to breast cancer susceptibility. The development of technology to specifically excise insertion mutations, which in this case would restore proper tumor suppressor activity, could be of considerable importance for the development of new, molecular based genetic therapeutics.

BODY

Task #1: Develop and analyze ribozymes that excise BRCA1 and p53 insertion mutations *in vitro*.

a. Develop and synthesize RNAs that mimic mutant mRNAs.
b. Develop and synthesize ribozymes that can remove these mutations.

We engineered two catalytic RNA ribozymes, one designed to specifically bind and excise insertion mutations from within a mutant BRCA1 transcript, and one specific for a mutation in a p53 transcript. The ribozymes were engineered via site-directed mutagenesis of a plasmid containing the *P. carinii* ribozyme by altering the recognition elements such that they base pair with the sequences flanking the desired BRCA1 and p53 mutations.

In addition, we synthesized small RNA mimics of the BRCA1 and p53 transcripts, centering on those regions flanking the mutations that are known to predispose individuals to breast cancer. In combination with one another, the ribozyme-transcript mimic combinations should change the 'Mutant' RNAs, as shown below, to the 'Fixed' RNAs.

U insertion in codon 2731 of BRCA1gene
Normal GCU CCG UUU - Ala - Pro - Phe - - -
Mutant GCU CCU **g**UU U - Ala - Pro - Gly → Stop
Fixed GCU CCU UUU - Ala - Pro - Phe - - -
End Result: Remove g (bold) and restore reading frame.

U insertion in codon 257 of p53 gene
Normal ACA CUG GAA - Thr - Leu - Glu - - -
Mutant ACA CUU **g**GA A - Thr - Leu - Gly → Stop
Fixed ACA CUU GAA - Thr - Leu - Glu - - -
End Result: Remove g (bold) and restore reading frame.

c. *Develop and perform assays in vitro to test for the excision of the designated mutations.*

Based on previous *in vitro* analysis of generic trans excision-splicing (TES) ribozymes (1-3), we developed and conducted excision reactions using the BRCA1 and p53 systems. In these cases, we radiolabeled the transcript mimics and followed the reactions by visualization on polyacrylamide gels, using migration distance as a marker for product size. Unfortunately, the BRCA1 specific ribozyme was not able to cut out the BRCA1 mutation in this *in vitro* system, even though many sequence variants of the ribozyme were analyzed. Fortunately, as can be seen in Figure 1, the 18 nucleotide p53 transcript mimic becomes the expected 17-mer product when the p53-specific ribozyme is added. In the optimized case, at 1 hour in 2 mM $MgCl_2$, approximately 50% of the transcript mimics are corrected via the excision of the breast cancer causing mutation.

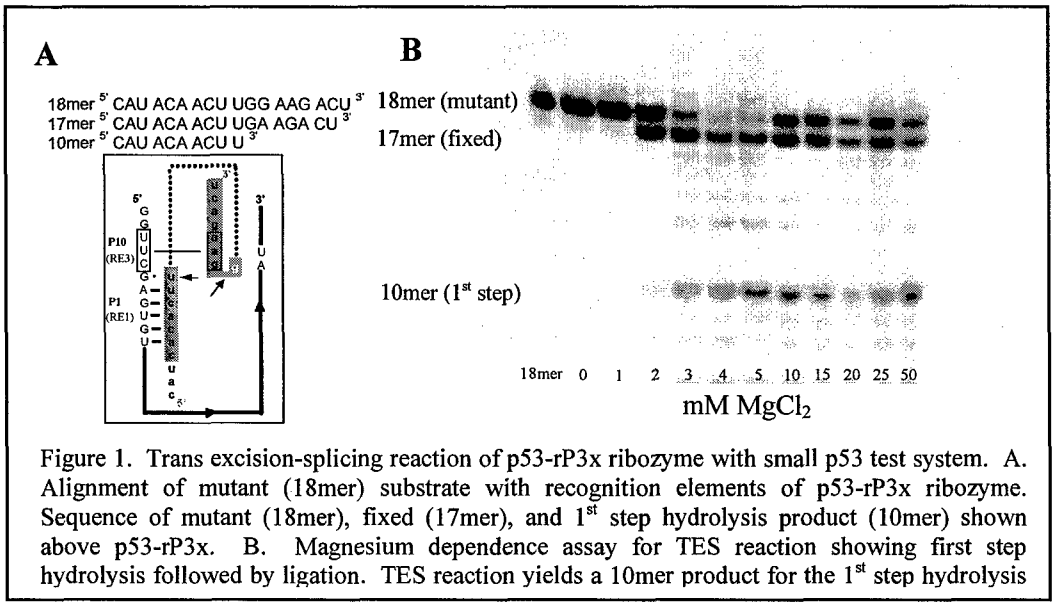

Figure 1. Trans excision-splicing reaction of p53-rP3x ribozyme with small p53 test system. A. Alignment of mutant (18mer) substrate with recognition elements of p53-rP3x ribozyme. Sequence of mutant (18mer), fixed (17mer), and 1st step hydrolysis product (10mer) shown above p53-rP3x. B. Magnesium dependence assay for TES reaction showing first step hydrolysis followed by ligation. TES reaction yields a 10mer product for the 1st step hydrolysis

We originally proposed two model systems for the *in vitro* studies (BRCA1 and p53), to enhance our chances that one system would work. This turned out to be wise, as the BRCA1 ribozyme appears to be inactive. However, the encouraging data on the p53 system lead us to continue these studies in this system (only). Although we attempted to analyze this reaction in a complete p53 transcript, the data were too difficult to interpret. The reason is that such a long mutant transcript will only differ from the fixed version by a single nucleotide, and so we could not visualize the difference on a polyacrylamide gel (as done above), and RT-PCR gave inconclusive results. We therefore decided that we needed a visual marker for transcript correction, ultimately at the protein (translated) level. Because the ultimate goal is to get the ribozyme to work *in vivo*, we designed a system whereby correction of the mutation would lead to cell fluorescence. This would therefore simultaneously address the question of *in vivo* reactivity, ribozyme specificity for the particular mutation, and ribozyme ability to target a a full-length RNA transcript. This strategy is outlined below in Task #2.

Task #2 Develop and analyze ribozymes that excise BRCA1 and p53 insertion mutations *in vivo*.

a. *Develop and synthesize shuttle vectors for expressing mutant genes and the repair ribozymes in the bacterium E. coli.*
b. *Develop and perform assays in vivo to test for the excision of the designated mutations in this cell system.*

To develop the *in vivo* system to test excision-repair, we first analyzed a ribozyme that would fix a single base insertion mutation in green fluorescent protein (GFP) *in vivo*. In this way, we can analyze *in vivo* excision, as it is tied with fluorescence as a visual marker. The experimental details and results are given in the manuscript (submitted) in the appendix. This manuscript outlines the first ever excision of a single base mutation from full-length transcripts (in this case GFP) *in vivo*, which now permits us to test the p53-specific ribozyme. Note that the same plasmids can be used to test ribozyme activity in mammalian cells, as we also have a complementary plasmid that expresses T7 RNA polymerase in mammalian cells.

In order to test whether the p53-specific ribozyme can excise out the particular cancer-causing mutation, we have linked a small portion of the p53 gene with the GFP gene, such that when the breast cancer mutation is in the p53 region of the transcript, GFP is inactivated and the cell does not fluoresces. After trying many GFP insertion sites unsuccessfully, we found that inserting the mutant p53 region between amino acids 172 and 173 of GFP still allowed GFP fluorescence. When the ribozyme is expressed and the cancer-causing mutation is excised, GFP is then re-activated, and success in the reaction can be seen and quantified via cellular fluorescence. A simple diagram of this idea is shown below in Figure 2. We have successfully synthesized such plasmids, containing both the p53-GFP 'fusion' transcript and the p53 specific ribozyme, and are about to test their *in vivo* activity.

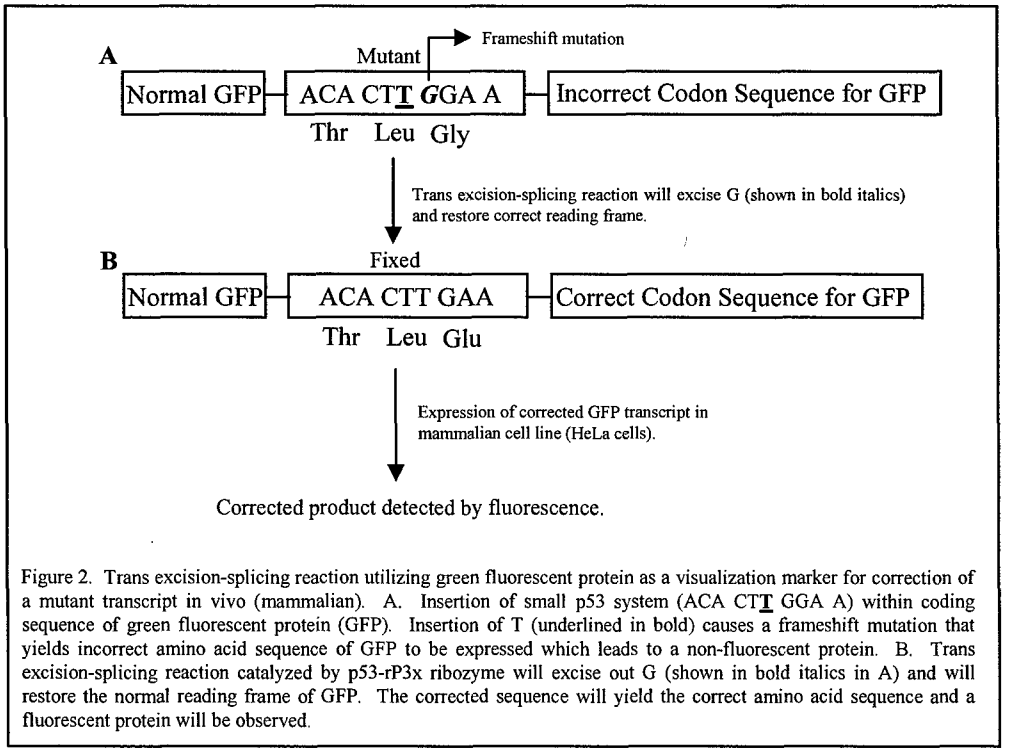

Figure 2. Trans excision-splicing reaction utilizing green fluorescent protein as a visualization marker for correction of a mutant transcript in vivo (mammalian). A. Insertion of small p53 system (ACA CTT GGA A) within coding sequence of green fluorescent protein (GFP). Insertion of T (underlined in bold) causes a frameshift mutation that yields incorrect amino acid sequence of GFP to be expressed which leads to a non-fluorescent protein. B. Trans excision-splicing reaction catalyzed by p53-rP3x ribozyme will excise out G (shown in bold italics in A) and will restore the normal reading frame of GFP. The corrected sequence will yield the correct amino acid sequence and a fluorescent protein will be observed.

A change to the original proposal is that two of the Ph.D. students have graduated and have been replaced on the project with new Ph.D. students. Another change, which does not affect the 'Statement of Work', is that GFP-p53 hybrid constructs will be used as cellular targets, because linking p53 transcript repair to cellular fluorescence allows for direct visualization of proper ribozyme activity. This has greatly facilitated our ability to conduct and analyze the *in vivo* experiments.

KEY RESEARCH ACOMPLISHMENTS

- The development, synthesis, and successful analysis of a catalytic RNA ribozyme system that can excise the U257 insertion mutation, which is a known predisposition indicator for breast cancer, from a small model mimic of the p53 transcript in a cell-free system.
- The optimization of reaction conditions for this ribozyme activity in a cell-free system.
- The development, synthesis, and successful analysis of a catalytic RNA ribozyme test system that can excise an insertion mutation from Green Fluorescent Protein, which provides for a visual marker of *in vivo* transcript repair.
- The development and synthesis of a catalytic RNA ribozyme system for analyzing the potential of a p53-specific ribozyme to excise the U257 insertion mutation from a GFP-p53 hybrid transcript *in vivo*.

REPORTABLE OUTCOMES

1) Manuscripts:
    i. See Appendix for submitted manuscript.

2) Seminar Presentations:
    i. "Exploiting the Properties of Nucleic Acids for the Development of Novel Strategies in Biotechnology," Chemistry Departmental Seminar, University of Kentucky, Lexington, KY, November 7, 2003.
    ii. "Developing New Intron-Derived RNA Catalysts: Potential Therapeutic Strategies," Markey Cancer Center Weekly Tumor Board Conference, (Put on by the Health Sciences Learning Center - Presentation is viewable from their web site), University of Kentucky, Lexington, KY, February 5, 2004.
    iii. "Strategies for Targeting RNA Transcripts," Markey Cancer Center Experimental Therapeutics Program Seminar, Universityof Kentucky, Lexington, KY, April 23, 2004.
    iv. "Nucleic Acids, Disease, and You," Department of Chemistry, University of Dayton, Dayton, OH, October 15, 2004.
    v. "Nucleic Acids, Disease, and You," Department of Chemistry, The University of The South, Sewanee, TN, October 28, 2004 (11:00 am).

vi. "Nucleic Acids, Disease, and You," Department of Chemistry, Berry College, Mt. Berry, GA, October 28, 2004 (5:00 pm).
vii. "Exploiting the Properties of Nucleic Acids for the Development of Novel Strategies in Biotechnology," Chemistry Department Seminar, Pennsylvania State University, State College, PA, November 9, 2004.

3) Degrees awarded partially supported by this award:
   i. 2 doctorates obtained

4) Funding applied for based on work supported by this award:
   i. National Institutes of Health "Mechanisms of Group I Intron Ribozyme Reactions", pending.

5) Employment opportunities applied for and/or received based on training supported by this award:
   i. 2 postdoctoral fellowships obtained

CONCLUSIONS

A wide variety of heritable genetic mutations that predispose individuals to particular cancers are known. Insertion mutations, for example, cause frameshift mutations that often lead to the synthesis of inactive and truncated proteins. At least 25 known insertion mutations have been identified in the genes BRCA1 and p53, which have been linked to breast cancer susceptibility. Each mutation impairs the essential functionality of the resultant tumor suppressor protein products. The development of technology to specifically excise these mutations, thus restoring proper tumor suppressor activity, would be of considerable importance for the development of new, molecular based genetic therapeutics. Our research directly addresses this problem by developing and testing a novel therapeutic strategy for the specific removal of RNA transcript mutations that have been linked to breast cancer predisposition. Using a short transcript model of the p53 gene in a cell free system, we have been able to design a molecule that can excise an insertion mutation that is known to predispose one to breast cancer. Using a model fluorescing system, we have been able to design a molecule that can specifically excise an insertion mutation from a full-length transcript in a simple bacterium. This ribozyme is not toxic to the cell, and appears to work with some degree of specificity. Although there is much work to be done, including targeting p53 transcripts, or mimics thereof, *in vivo* and in mammalian cells, we have developed the first molecules (of any kind) that can target insertion mutations in a cell and specifically excise them, thus repairing the transcripts. This work brings the idea of fixing insertion mutations that lead to genetic disease into the realm of possibility, and will be the basis for many translational research projects.

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3) Baum, D. A., Sinha, J., and Testa S. M. (2005) "Molecular Recognition in a Trans Excision-Splicing Ribozyme: Non-Watson-Crick Base Pairs at the 5' Splice Site and ωG at the 3' Splice Site Can Play a Role in Determining the Binding Register of Reaction Substrates" *Biochemistry* 44, 1067-1077.

APPENDIX

In Vivo Excision of a Single Targeted Nucleotide from an mRNA by a Trans Excision-Splicing Ribozyme

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Running Title: *In Vivo* Trans Excision-Splicing Reaction

Key words: Trans excision-splicing; ribozyme; RNA repair

ABSTRACT

We have previously reported the development of a group I intron-derived ribozyme that can bind an exogenous RNA substrate and excise from that substrate an internal segment *in vitro*, which allows for sequence-specific modification of RNA molecules. In this report, the activity of this trans excision-splicing ribozyme in a cellular environment, specifically *E. coli*, was investigated. The ribozyme was re-engineered to target for excision a single-base insertion in the transcript of a green fluorescent protein, and fluorescence was exploited as a reporter for trans excision-splicing. We show that the ribozyme is able to catalyze the trans-excision splicing reaction *in vivo* and can repair upwards of 12% of the mutant transcripts, as measured by fluorescence and confirmed by sequencing of isolated transcripts. This represents the first report of a biomolecule (in this case a ribozyme) that can selectively excise a targeted nucleotide from within an mRNA transcript *in vivo*. This new class of biochemical tools makes possible a wide variety of new experimental strategies, perhaps including a new approach to molecular-based therapeutics.

INTRODUCTION

Group I introns are catalytic RNAs (Kruger et al. 1982) with the ability to splice themselves out of RNA transcripts. Eliminating the 5' and 3' exon sequences from these self-splicing introns produces catalytic ribozymes that are able to bind substrates in *trans* and perform reactions analogous to the reaction steps of self-splicing (Zaug et al. 1986; Zaug and Cech 1986; Cech 1990; Sargueil and Tanner 1993; Testa et al. 1997). Furthermore, such ribozymes have been developed with the ability to target and modify mRNA transcripts for biochemical and therapeutic purposes (Sullenger and Cech 1994; Jones et al. 1996; Jones and Sullenger 1997; Phylactou et al. 1998; Kohler et al. 1999; Rogers et al. 2002; Byun et al. 2003; Ryu et al. 2003; Kastanos et al. 2004; Shin et al. 2004). For example, it was previously reported that a ribozyme derived from a *Tetrahymena* group I intron catalyzes a trans-splicing reaction (Sullenger and Cech 1994), in which the ribozyme binds an RNA substrate and replaces its defective 3' exon sequence with a corrected form (carried by the ribozyme). The *in vivo* activity of this ribozyme has been demonstrated with various transcript targets (Sullenger and Cech 1994; Jones et al. 1996; Jones and Sullenger 1997; Phylactou et al. 1998; Kohler et al. 1999; Rogers et al. 2002; Byun et al. 2003; Ryu et al. 2003; Kastanos et al. 2004; Shin et al. 2004). This type of RNA modification has potential therapeutic application as it can be used to repair transcripts with mutations that lead to disease.

A complementary reaction to trans-splicing is the trans excision-splicing (TES) reaction (Bell et al. 2002). In this reaction, a group I intron-derived ribozyme from *Pneumocystis carinii* binds an RNA substrate and excises a targeted segment from within that substrate (Figure 1). *In vitro*, this ribozyme has been used to excise inserts from 1 to 28 nucleotides in length and can be reengineered to target non-native sequences, including the flanking region of the trinucleotide repeat that causes Myotonic Dystrophy (Bell et al. 2002; Bell et al. 2004). The usefulness of these ribozymes, however, will be greatly enhanced by demonstrating *in vivo* reactivity.

In this report, we have designed a test system to assess the ability of the *P. carinii* ribozyme to catalyze the TES reaction *in vivo*. A single base insertion mutation was engineered into a green fluorescent protein (GFP) gene, which consequently destroys fluorescence of the resultant protein product. The TES ribozyme was then reengineered to target this mutation, so a successful TES reaction reestablishes the reading frame of the protein in the GFP transcript

(Figure 2A), producing a fluorescing protein and thus a fluorescing cell culture. We now report that the ribozyme is able to catalyze the TES reaction in *E. coli*. Upwards of 12% correction was observed using a construct with an elongated P10 helix of 5 base pairs, indicating an *in vivo* requirement for strong interactions between the ribozyme and the target transcript. Sequencing of isolated transcripts confirmed the ribozyme-mediated removal of the targeted nucleotide. This represents the first report of a biomolecule (in this case a ribozyme) that can selectively excise a targeted nucleotide from within an mRNA transcript *in vivo*. This new class of biochemical tools makes possible a wide variety of new experimental strategies, perhaps including a new approach to molecular-based therapeutics.

RESULTS

Design of the in vivo test system. The design of the *in vivo* TES test system was based on work involving the trans-splicing ribozyme (Sullenger and Cech 1994; Jones et al. 1996; Jones and Sullenger 1997; Phylactou et al. 1998; Kohler et al. 1999; Rogers et al. 2002; Byun et al. 2003; Ryu et al. 2003; Kastanos et al. 2004; Shin et al. 2004), as well as design principles garnered through studies of the TES ribozyme *in vitro* (Bell et al. 2002; Bell et al. 2004). Our simplest TES reaction system involves the removal of a single nucleotide from a substrate, so we designed a test system in which we create a single base insertion mutation in the coding region of GFP (Figure 2A). This mutation causes a deleterious shift in the reading frame of the transcribed RNA, which alters the amino acid sequence of the resultant protein product and creates a premature stop codon, resulting in a loss of fluorescence. We chose to insert a uridine 5' to a guanosine in the RNA transcript (Figure 2A). In the context of the TES reaction, this uridine will serve to define the 5' splice site by forming the highly conserved u-G wobble pair with a guanosine in the ribozyme (Barfod and Cech 1989; Doudna et al. 1989; Testa et al. 1997; Bell et al. 2002). The guanosine following the inserted uridine is equivalent to the ω position in the self-splicing reaction (Price and Cech 1988; Burke 1989; Michel et al. 1989; Burke et al. 1990; van der Horst and Inoue 1993), so this ωG is the base targeted for excision (Figure 2B). After a successful TES reaction, the inserted uridine replaces the guanosine in the coding region, resulting in a silent mutation in the restored reading frame (Figure 2A). Thus, the site for insertion was limited to those codons where replacement of guanosine by uridine results in the same amino acid when translated into protein. Codon 201, which codes for leucine, was an attractive target for insertion and, based on RNA structure prediction estimates, was accessible in the transcript. The insertion of a uridine and subsequent removal of a guanosine allows us to determine, via the sequence of isolated transcripts from our test reactions, that the fluorescent form of GFP results from a successful TES reaction.

Changes to modify the ribozyme were kept to a minimum for these initial *in vivo* studies and involved altering the recognition elements of the ribozyme, which are responsible for target identification (Figure 2B). In all the test systems, the length of recognition element 1 (RE1) was maintained at 6 bases. Previous work on the TES ribozyme indicated that increasing the length of RE3 can be beneficial to the reaction (Bell et al. 2004), while work with the trans-splicing ribozyme showed a requirement for increased 3' exon interactions for *in vivo* activity (Kohler et al. 1999; Rogers et al. 2002; Byun et al. 2003; Ryu et al. 2003; Kastanos et al. 2004; Shin et al. 2004). Thus, ribozymes were tested with RE3 lengths of 3 bases (native length), 5 bases, and 7 bases. A third recognition element, RE2, is not utilized in TES reactions that remove a single nucleotide, so it was not modified. As a control for ribozyme activity, we also created mutant

ribozyme constructs by deleting four bases from the guanosine binding site of the ribozyme. This mutation completely inactivates the ribozyme when tested *in vitro* (data not shown).

The TES ribozyme is active in vivo. First, the insertion mutation engineered into the GFP target was tested to ensure that it did indeed abolish fluorescence. The expected corrected form (with the G-to-U transversion) was also tested for fluorescent activity. As seen in Figure 3, introducing the insertion mutation into the GFP gene abolishes GFP activity. The expected corrected form fluoresces, albeit at a level somewhat below that for the normal form of GFP, indicating the G-to-U transversion is not substantially disruptive to the translation of the GFP protein.

Next, the ribozymes engineered to target the mutation in GFP were tested for *in vivo* activity by pairing the ribozymes with the mutant form of GFP (Mut GFP). Targeted ribozymes (GFP rP3X) were tested with RE3 lengths of 3 bases, 5 bases and 7 bases. Increasing the length of RE3 increases the interaction between the ribozyme and the 3' exon of the substrate, which helps prevent 3' exon dissociation prior to the second reaction step and can improve TES product formation *in vitro* (Bell et al. 2004). The native form of the ribozyme (rP3X) served as a non-GFP-targeted control for ribozyme activity, while inactive forms of the ribozymes (containing a 4-base deletion in the guanosine binding site) were used as negative controls. As seen in Figure 4, increasing the length of RE3 to 5 bases for the targeted ribozyme (GFP rP3X RE3=5) significantly increases the fluorescence over the inactive ribozyme controls, indicating TES reactivity. Note the level of fluorescence for the negative controls is not zero due to a low level of inherent fluorescence in the samples. We obtained an estimate of TES reactivity by comparing the data from the constructs with mutant GFP (Figure 4) and the same ribozyme constructs with the expected corrected form of GFP. For the corrected forms of GFP, normalized fluorescence values of 16265.99 ± 388.75 (ribozyme with an RE3=3), 3105.83 ± 1940.59 (ribozyme with an RE3=5), and 5971.97 ± 2272.05 (ribozyme with an RE3=7) were obtained from at least 3 independent tests. Note the large standard deviations result from the expected variability in reactions conducted *in vivo*. The estimated percentage of RNA repair was low for the targeted ribozymes with RE3 lengths of 3 nucleotides (1.09%) and 7 nucleotides (2.84%), while utilizing a targeted ribozyme with an RE3 length of 5 nucleotides increased the estimated percentage of repair to 12.08%.

Sequence confirmation of in vivo TES reaction. Total RNA was isolated from reactions involving 3 different constructs: the mutant GFP paired with the targeted ribozyme containing an RE3=5 (the repair construct that showed the greatest increase in GFP activity), the mutant GFP paired with the inactive targeted ribozyme with an RE3=5 (a negative control), and the expected corrected form of GFP paired with the targeted ribozyme with an RE3=5 (a positive control to assess the insertion efficiency). The total RNA was subjected to RT-PCR to isolate the GFP transcript and the resulting RT-PCR products were ligated into a cloning vector and were assayed for their ability to produce fluorescent protein (Figure 5). In the screening assays, twelve fluorescent colonies (out of ~2000 screened colonies) were obtained from products from 2 independent *in vivo* tests with the active repair construct, indicating the presence of corrected transcripts. Assays involving products from three independent *in vivo* tests with the mutant ribozyme construct produced no fluorescent colonies in ~2000 screened colonies, indicating that no TES reaction had occurred. The twelve fluorescent colonies from the active repair construct, one non-fluorescent colony from the active repair construct and eighteen colonies from the negative control construct were isolated and the resulting plasmids were sequenced. All twelve sequences from the fluorescent colonies showed the removal of the targeted guanosine, resulting

in the G-to-U transversion in the corrected transcript (Figure 6). If the cell randomly fixed the transcript and produced fluorescent protein, the loss of a uridine would be anticipated to be as likely as the loss of a guanosine. As this loss of a uridine was not observed, the fluorescent protein was produced as a result of a successful TES reaction. There was also no evidence of a deletion that could serve as a compensatory mutation to restore fluorescence. The non-fluorescent colonies were confirmed to contain the unrepaired mutant GFP transcript (Figure 6). Thus the removal of the guanosine was TES ribozyme-mediated and the ribozyme is sequence-specific for its target, without being deleterious to the cell.

DISCUSSION

The arsenal of molecular-based RNA tools is rapidly growing, however, the majority of these tools have focused on the destruction of the RNA message rather than the modification of the message (Sullenger and Gilboa 2002; Puerta-Fernandez et al. 2003; Scherer and Rossi 2003). Destroying the RNA message is useful for studying the effects of shutting off genes and can be useful in therapeutic applications to prevent the production of mutant proteins that lead to disease. On the other hand, modifying the message can restore the function to the transcript, thus reducing mutant protein and producing normal protein. With this in mind, we report the development of a catalytic RNA that can sequence-specifically target a single nucleotide within a cellular transcript *in vivo* and excise that targeted nucleotide from a transcript, with little or no apparent toxicity to the cell. Combined with the ease of adaptability, this provides for a wide variety of new *in vivo* experimental strategies.

As biochemical tools, TES ribozymes could be used as inducible tools to modulate the production of proteins *in vivo*. As demonstrated in this report, TES ribozymes can be targeted to remove sequences to restore the reading frame of a transcript, thus producing active protein. The removal of sequences to shift the sequence out of frame is also possible, so inactive or mutant proteins could be produced in an inducible manner.

The applications of TES ribozymes could also include therapeutic applications. As a therapeutic agent, TES ribozymes could be used to remove insertion mutations and premature stop codons from transcripts, thus restoring the reading frame of the transcript for protein production. We have previously demonstrated the *in vitro* ability of a TES ribozyme to target a small model mimic of the triplet expansion implicated in Myotonic Dystrophy and to excise from that mimic a short triplet expansion (Bell et al. 2002). Thus the repair of transcripts involved in triplet expansion diseases, such as Muscular Dystrophy and Huntington's Disease, is another potential application for TES ribozymes *in vivo*.

As TES ribozymes recognize their targets initially and primarily through base pairing, engineering the ribozymes to target new transcript regions simply requires changing the sequence of the recognition elements to base pair with the desired target. Other considerations for ribozyme targeting include reconstituting a u-G wobble pair at the 5' splice site and a guanosine at the 3' splice site as the last (or only) base of the sequence targeted for excision (Baum et al. 2004). Also note that previous work with this ribozyme demonstrated that segments larger than a single nucleotide can be excised *in vitro* (Bell et al. 2002), so we anticipate that larger regions could be excised *in vivo*.

Comparing in vivo and in vitro TES results. In our simplest test system, which involves the excision of a single, targeted nucleotide, the TES ribozyme is able to produce 70% product *in vitro* under optimized conditions of ribozyme excess and 10 mM $MgCl_2$ (Bell et al. 2002). For

the *in vivo* tests presented here, the ribozyme produces approximately 12% product, based on fluorescence data. This decrease in reactivity is not unexpected and is likely due to a number of factors, including a lower concentration of magnesium, a lower concentration of active ribozyme relative to the target substrate, and target accessibility. Nevertheless, TES ribozymes are adaptable to the cellular environment and are able to produce detectable amounts of repaired RNA.

Comparison with trans-splicing and SMaRT. TES ribozymes offer a different approach to RNA modification compared to the *Tetrahymena*-derived trans-splicing ribozyme (Sullenger and Cech 1994) and spliceosome-mediated RNA *trans*-splicing, or SMaRT (Puttaraju et al. 1999; Garcia-Blanco 2003). The trans-splicing ribozyme replaces the 3' exon of a targeted transcript with a new version of the exon, which is carried by the ribozyme. SMaRT utilizes the spliceosomes present in the cell to catalyze the splicing between an exon on the target transcript and an exon present in a pre-trans-splicing molecule (PTM). TES ribozymes are different in that they excise a targeted internal segment from the transcript.

TES ribozymes have some potential advantages over trans-splicing ribozymes and SMaRT. TES ribozymes are not changed in the reaction, so they have the potential to be multiple-turnover catalysts. The trans-splicing ribozyme splices its replacement exon into the target, making it single turnover. The PTM donates its replacement sequence during splicing, so it also is not a multiple turnover reaction. TES ribozymes can, in theory, be engineered to excise an internal segment anywhere on a transcript. In contrast, trans-splicing ribozymes are best suited to target mutations in the 3' portion of a transcript and SMaRT targets only exon/intron junctions. In addition, TES ribozymes do not carry replacement sequences, while trans-splicing ribozymes and PTMs do. Therefore, the size of the ribozyme-replacement exon construct or the PTM could become prohibitive for effective delivery and activity. Finally, in contrast to TES ribozymes and trans-splicing ribozymes, SMaRT can only be used in mammalian cells as it requires spliceosomes.

In *E. coli*, the level of activity restored by the trans-splicing ribozyme was approximately 1% (Sullenger and Cech 1994). In our studies, we observe a higher level of repair in *E. coli*, as the level of activity restored by the TES ribozyme was approximately 12%. SMaRT is limited to mammalian cells, so we cannot accurately compare the levels of product formation to our *E. coli* results. That trans-splicing ribozymes (Sullenger and Cech 1994; Jones et al. 1996; Jones and Sullenger 1997; Phylactou et al. 1998; Kohler et al. 1999; Rogers et al. 2002; Byun et al. 2003; Ryu et al. 2003; Kastanos et al. 2004; Shin et al. 2004) and SMaRT (Puttaraju et al. 1999; Liu et al. 2002; Chao et al. 2003) both work fairly well in mammalian cells (upwards of 50%) is encouraging, and we anticipate that TES ribozymes will also be active in mammalian systems. As is the case with trans-splicing (Jones and Sullenger 1997; Rogers et al. 2002; Byun et al. 2003; Shin et al. 2004) and SMaRT (Liu et al. 2002; Chao et al. 2003), we do not anticipate that TES modification will have to be 100% to have measurable effects as a biochemical tool. A low level of TES activity may produce a relevant amount of modified transcript for protein production.

Note that the challenges we face in improving the TES reaction *in vivo* and adapting TES ribozymes to a mammalian system are the same challenges faced by others developing ribozymes in mammalian systems (Sullenger and Gilboa 2002; Long et al. 2003; Scherer and Rossi 2003). Common issues in *E. coli* and mammalian cells include increasing the level of ribozyme activity, improving ribozyme specificity, assessing target accessibility, and effectively delivering the ribozyme. Work is ongoing to address these issues and is encouraging (Sullenger

and Gilboa 2002; Long et al. 2003; Puerta-Fernandez et al. 2003; Scherer and Rossi 2003). In conclusion, this study demonstrates the potential of TES ribozymes as new tools for the *in vivo* sequence-specific modification of RNA transcripts.

MATERIALS AND METHODS

Oligonucleotide synthesis and preparation. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Oligonucleotides for the insertion of the T7 terminator sequence were 5' phosphorylated and PAGE purified by the company. Oligonucleotides for site-directed mutagenesis and RT-PCR were used without further purification.

Plasmid Construction. The *P. carinii* ribozyme precursor plasmid was constructed as previously described (Testa et al. 1997). To stop transcription of the ribozyme *in vivo*, a T7 terminator sequence (Studier et al. 1990) was inserted immediately 3' to the ribozyme sequence using the *Xba*I and *Hind*III sites present in the P3X plasmid (Figure 7). The ligation product was used to transform *Escherichia coli* DH5α competent cells (Invitrogen, Carlsbad, CA). The resulting plasmid (P3X+T7T) was purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and sequenced for confirmation (ACGT, Inc., Northbrook, IL).

Two unique restriction sites, *Bgl*II and *Sph*I, were engineered into the newly created P3X+T7T plasmid to allow for the excision of the ribozyme construct (Figure 7). The plasmid, P3X+T7TBS, was created by 2 successive rounds of site-directed mutagenesis. The following primer pair was used to create a *Bgl*II restriction site upstream of the T7 promoter: $^{5'}$GGAAACAGATCTGACATGATTACGAATTTGG$^{3'}$ and $^{5'}$CCAAATTCGTAATCATGTCAGATCTGTTTCC$^{3'}$. A *Sph*I restriction site was created downstream of the T7 terminator with the following primer pair: $^{5'}$GCTTACTAGTGATGCATGCTCTATAGTGTCACC$^{3'}$ and $^{5'}$GGTGACACTATAGAGCATGCATCACTAGTAAGC$^{3'}$. The site-directed mutagenesis reactions were conducted as previously described (Bell et al. 2002), with the changes that follow. The reaction mixtures were subjected to denaturation at 95 °C for 30 s, followed by 15 temperature cycles of 95 °C for 30 s, 55 °C for 2 min, and 68 °C for 6 min. The parental plasmids were digested with 20 units of *Dpn*I (New England Biolabs, Beverly, MA) in 4.2 μL of manufacturer's buffer for at least 1 h at 37 °C. A 3-μL aliquot of this digest was then used to transform *E. coli* DH5α competent cells (Invitrogen). The resultant plasmids were purified as above and were sequenced for confirmation (Davis Sequencing, Davis, CA).

The plasmid containing the GFP target, pQBI T7-GFP, was purchased from Qbiogene (Carlsbad, CA). The base plasmid for testing, pQBI GFP + P3X, was created by inserting the ribozyme construct into the pQBI T7-GFP plasmid using the *Bgl*II and the *Sph*I restriction sites present in the pQBI T7-GFP plasmid (Figure 7). The ribozyme construct was isolated by digesting the P3X+T7TBS plasmid with *Bgl*II and *Sph*I. The ligation product was used to transform *E. coli* DH5α competent cells (Invitrogen). The resulting plasmid was purified and sequenced as above.

Test plasmids were generated via site-directed mutagenesis of the base plasmid pQBI GFP + P3X. The following primers were used to modify the GFP gene to contain a single base insertion mutation to destroy GFP fluorescence (Mut GFP): $^{5'}$GCAGATTGTGTGGACAAGGTAATGGTTGTCTGG$^{3'}$ and $^{5'}$CCAGACAACCATTACCTTGTCCACACAATCTGC$^{3'}$. The underlined base represents the

insertion mutation. The corrected form of GFP expected from a successful TES reaction (Corr GFP) was created with the following primers:
$^{5'}$GCAGATTGTGTGGAAAGGTAATGGTTGTCTGG$^{3'}$ and
$^{5'}$CCAGACAACCATTACCTTTCCACACAATCTGC$^{3'}$. The underlined base represents the silent mutation expected from removal of the targeted guanosine by the TES ribozyme. These mutations alter codon 201 of the GFP gene. Site-directed mutagenesis was performed as above, with the following modifications. The reaction mixtures were subjected to denaturation at 95 °C for 30 s, followed by 15 or 18 temperature cycles of 95 °C for 30 s, 50 °C or 55 °C for 2 min, and 68 °C for 6 min or 8 min, depending on the construct. Resulting plasmids were purified and sequenced as above.

The ribozyme portion of the test plasmid was modified in the following ways using site-directed mutagenesis. First, the recognition elements of the ribozyme (RE1 and RE3) were modified to recognize the GFP target by two successive rounds of site-directed mutagenesis. A third recognition element, RE2, is not utilized in TES reactions involving the removal of a single nucleotide, so it was not modified. The primers for the first round were:
$^{5'}$CGACTCACTATAGAGGGGGTAGAAAGCGGC$^{3'}$ and
$^{5'}$GCCGCTTTCTACCCCCTCTATAGTGAGTCG$^{3'}$. The second round of changes was made with the following primers: $^{5'}$CGACTCACTATAGGAGAGGTAGAAAGCGGC$^{3'}$ and
$^{5'}$GCCGCTTTCTACCTCTCCTATAGTGAGTCG$^{3'}$. The length of RE3 was increased to 5 bases using the following primers: $^{5'}$CGACTCACTATAGTGGAGAGGTAGAAAGCGGC$^{3'}$ and $^{5'}$GCCGCTTTCTACCTCTCCACTATAGTGAGTCG$^{3'}$. An RE3 containing 7 bases was created using the following primer pair:
$^{5'}$CGACTCACTATAGTGTGGAGAGGTAGAAAGCGGC$^{3'}$ and
$^{5'}$GCCGCTTTCTACCTCTCCACACTATAGTGAGTCG$^{3'}$. A mutant form of the ribozyme was created by deleting four bases (bases 250-253) from the guanosine binding site of the ribozyme (Testa et al. 1997). Site-directed mutagenesis reactions were performed as above and the resulting plasmids were sequenced for confirmation (Davis Sequencing).

Preparing competent JM109(DE3) and transforming competent JM109(DE3). In vivo testing of the TES ribozyme was conducted in *E. coli* strain JM109(DE3) (Promega, Madison, WI). Competent JM109(DE3) cells were prepared using the rubidium chloride protocol reported by Promega (Doyle 1996). Following a 1 h incubation on ice, the competent cells were transformed with the test plasmids using a modified version of the procedure outlined by Promega (Doyle 1996). 100 µL of competent cells was used in each transformation reaction. Approximately 10 ng of test plasmid was added to the competent cells and the tubes were incubated for 30 min on ice. The tubes were then heated at 44 °C for 50 s and placed immediately back on ice for 2 min. 1.5 mL of LB media was then added to each tube and the tubes were incubated at 37 °C with shaking at 225 rpm for 45 min. After incubation, the transformations were plated on LB plates containing ampicillin and the plates were incubated at 37 °C overnight.

In vivo TES reactions. The colonies resulting from transformation of the competent JM109(DE3) cells were used for *in vivo* testing of the TES ribozymes. Colonies were isolated and grown up in liquid culture overnight. The following day, the overnight cultures were used to inoculate fresh LB media without antibiotic to create 1:20 dilution cultures. The dilution cultures were incubated at 37 °C with shaking at 225 rpm for 3 h to allow growth to reach log phase (A_{600} > 0.4). After 3 h, aliquots were removed from each culture for cell density analysis (500 µL) and fluorescence analysis (1 mL). The rest of the dilution culture was then subdivided. The inducer,

isopropylthiogalactoside (IPTG), was added to one of the cultures to a final concentration of ~1.3 mM, while the other culture served as a non-induced control. Cultures were incubated at 37 °C with shaking at 225 rpm for 5 h. After 5 h, aliquots were removed for cell density analysis (500 µL), GFP fluorescence analysis (1 mL) and for select cultures, total RNA isolation (1 mL).

Analysis. Cell density was determined by measuring the absorbance of the culture at 600 nm (A_{600}). For GFP fluorescence analysis, the culture aliquots were centrifuged to pellet the cells. The media was removed and the cell pellets were resuspended in 300 µL of phosphate buffered saline (PBS) at pH 7.4. The resuspended pellets were then loaded into a FluoroNunc™ MaxiSorp™ 96-well plate (Nalge Nunc International, Rochester, NY). GFP fluorescence was measured in a CytoFluor (PerSeptive Biosystems, Framingham, MA), using a 485±20 nm filter for excitation and a 508±20 nm filter for emission, as the excitation wavelength for this form of GFP is 474 nm and the emission wavelength is 509 nm. The raw fluorescence measurements were corrected for differences in cell culture growth by dividing the raw fluorescence values by the A_{600} readings. These corrected fluorescence values were then normalized to the non-induced values to correct for "leaky" T7 RNA polymerase activity by subtracting the corrected fluorescence for the non-induced culture from the corrected fluorescence for the induced culture. The reported normalized fluorescence values are the result of at least 3 independent assays. Note that omitting the corrections for differences in cell density does not significantly affect the trends obtained (data not shown).

Total RNA isolation. Total RNA was isolated using 1 mL aliquots from designated cultures using the Ambion RiboPure™-Bacteria Kit (Ambion, Inc., Austin, TX) and was treated with RQ1 RNase-Free DNase (Promega) to completely remove the DNA prior to RT-PCR. RNA was isolated from the DNase reaction mixture by acid phenol (pH 4.3) extraction, followed by phenol/chloroform extraction and ethanol precipitation.

RT-PCR reactions. The GFP transcripts were amplified from the total RNA by RT-PCR using the following primers: $^{5'}$GTTGTACAGTTCATCCATGCC$^{3'}$ and $^{5'}$GGAGAAGAACTCTTCACTGG$^{3'}$. RT-PCR reactions were performed using the Access RT-PCR System (Promega) and consisted of 50 µL reactions containing ~1 µg total RNA, 1 mM $MgSO_4$, 45 pmol of each primer, 0.2 mM dNTPs, 5 U AMV Reverse Transcriptase, and 5 U Tfl DNA Polymerase in the provided reaction buffer. PCR reactions were performed as a control to ensure the DNA had been removed from the samples. The reactions were subjected to 45 °C for 45 min for first strand cDNA synthesis, followed by 2 min at 94 °C to inactivate the AMV Reverse Transcriptase. The reactions then underwent 40 temperature cycles consisting of 94 °C for 30 s, 54 °C for 1 min, and 68 °C for 2 min. After cycling was complete, the reactions underwent a final extension cycle at 68 °C for 10 min. The RT-PCR products were separated on a 2% agarose gel and the GFP band was excised. The band was extracted from the gel matrix using a QIAquick Gel Extraction Kit (Qiagen). The gel-purified products were ethanol precipitated twice prior to use in ligation reactions.

Assay for corrected transcripts. The TES reaction removes a targeted sequence from within an RNA, so the 5' and 3' ends are the same in the mutant and corrected forms. Moreover, in our simple system, the difference between the mutant GFP and the corrected GFP is a single base. Thus, the product cannot trivially be selectively amplified by RT-PCR. Therefore, we designed an assay to analyze the pool of transcripts after the reaction for those that are corrected and thus produce fluorescent protein (Figure 5). The GFP RT-PCR products were ligated into the pDrive cloning vector using the QIAGEN PCR Cloning kit (Qiagen). Ligation reactions consisted of 50 ng pDrive cloning vector, 200 ng of RT-PCR product, 1 µL 50% PEG (5% final

w/v), and 2X Ligation Master Mix (provided with the kit) in a final volume of 10 μL. Ligations proceeded for 1.5 h at 4 °C. Following ligation, a 3-μL aliquot of the ligation reaction was used to transform *E. coli* DH5α competent cells (Invitrogen). Transformations were plated on LB media plates containing kanamycin and the plates were incubated at 37 °C overnight. The following day, the plates were placed at 4 °C for at least 4 h to enhance GFP fluorescence for visualization. Those colonies containing the corrected GFP products will produce fluorescent GFP, which can be visualized on the plate. After incubation at 4 °C, the plates were exposed to UV light and colonies showing GFP activity were isolated. The resulting plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen) and sequenced for confirmation (Davis Sequencing).

ACKNOWLEDGEMENTS

The authors wish to thank Rashada Alexander, Dustin Lafferty and Joy Sinha for technical assistance and members of the Testa lab for helpful discussion. This work was supported by the Muscular Dystrophy Association and the DOD Breast Cancer Research Program DAMD17-03-1-0329.

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FIGURE 1: Ribozyme Trans Excision-Splicing reaction. The TES ribozyme is shown as a gray line, the 5' and 3' exons are black lines and the sequence targeted for excision (in this case, a single guanosine) is a gray box. The circle in the 5' exon represents uridine. P1 and P10 are the helices that result from the ribozyme recognition elements RE1 and RE3 (respectively) base pairing with the substrate.

FIGURE 2: Design of the GFP target system (A) and Schematic of the two-step TES reaction utilizing the GFP target (B). (A) The top line shows the sequence of interest of the GFP gene. The bold codon codes for leucine (Codon 201). The gray box is the sequence that base pairs with recognition element 1 (RE1) of the ribozyme. Site-directed mutagenesis results in the insertion of a uridine (underlined) 5' to a guanosine in the mRNA transcript, which causes a frameshift and a premature stop codon (not shown). The TES reaction removes the targeted guanosine and restores the correct reading frame of the GFP gene (bottom line). (B) Diagram of the recognition elements of the TES ribozyme base pairing with the GFP target. The GFP rP3X ribozyme is in uppercase lettering, the GFP target is in lowercase lettering, and the guanosine to be excised is in white lettering. The ribozyme recognition elements RE1, RE2, and RE3 base pair with the substrate to form the P1, P9.0, and P10 helices, respectively. Note that the P9.0 helix is not utilized in this system. The sites of catalysis for the first step (5' cleavage) and the second step (exon ligation) are shown with large bold arrows. The 3-base P10 helix is boxed, with brackets indicating the extensions to 5 and 7 bases. Note that the diagram only shows the recognition elements of the ribozyme. This ribozyme is the same as rP-8/4x (Testa et al. 1997), except for the sequences of RE1 and RE3 and the addition of a T7 terminator on the 3' end.

FIGURE 3: Comparison of the normalized GFP fluorescence levels for the normal, mutant and corrected forms of GFP. All points utilize constructs with the non-targeted ribozyme and the GFP form listed on the graph. Normalized fluorescence was determined by correcting the raw fluorescence for differences in cell density (determined by the absorbance of the bulk culture at 600nm) and normalizing to non-induced controls. Each data point represents 4 independent assays and the standard deviations are below 10%.

FIGURE 4: Normalized fluorescence data for constructs with mutant GFP paired with the TES ribozymes. All points utilize the mutant GFP target with the ribozyme listed above the graph. The + symbols and solid bars indicate active ribozymes, while the – symbols and open bars indicate the ribozymes have been inactivated by a deletion in the guanosine binding site. Normalized fluorescence was determined by correcting the raw fluorescence for differences in cell density (determined by the absorbance of the bulk culture at 600nm) and normalizing to non-induced controls. Each data point represents 4 independent assays and the standard deviations are below 10%.

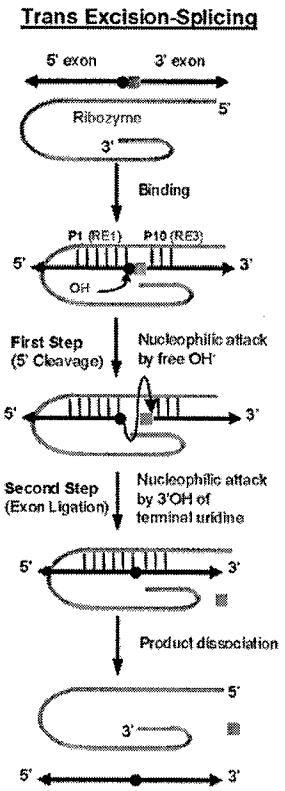
FIGURE 5: Schematic of the screening assay for isolation of corrected transcripts. Total RNA is isolated from cells that have undergone *in vivo* testing. The isolated RT-PCR products are ligated into the pDrive PCR cloning vector (Qiagen). Note that the RT-PCR products can be ligated into the vector in the forward direction (which puts the GFP gene in frame with the LacZ α-peptide of the plasmid) or the reverse direction, with each direction assumed to be equally possible. Only products ligated in the forward direction will product fluorescent GFP. The resulting plasmids are isolated and sequenced to confirm the expected sequence change in the corrected transcripts.

FIGURE 6: Sequencing confirmation of uncorrected GFP (left) and corrected GFP (right) transcripts. Total RNA isolated from *in vivo* tests involving the construct Mut GFP + GFP rP3X RE3=5 was utilized as the template for RT-PCR to isolate the GFP transcript.

FIGURE 7: Construction of the *In Vivo* TES Test Plasmid. The original TES ribozyme plasmid (P3X) was modified by the insertion of a linker containing a T7 terminator sequence 3' to the ribozyme sequence to create P3X + T7T. Site-directed mutagenesis was used to create a unique *Bgl*II site 5' to the T7 promoter of the ribozyme and a unique *Sph*I site 3' to the T7 terminator of the ribozyme (P3X + T7TBS). The ribozyme construct was isolated from the plasmid by a double restriction enzyme digest with *Bgl*II and *Sph*I. The GFP-containing plasmid, pQBI T7-GFP (Qbiogene), was prepared for insertion of the ribozyme construct by a double enzyme digest with *Bgl*II and *Sph*I. The ribozyme construct was ligated into the cut pQBI T7-GFP plasmid to create pQBI GFP + P3X. Modifications to the GFP gene to create the mutant and corrected forms and modifications to the ribozyme to change the recognition elements and to create the inactive forms were all created via site-directed mutagenesis.

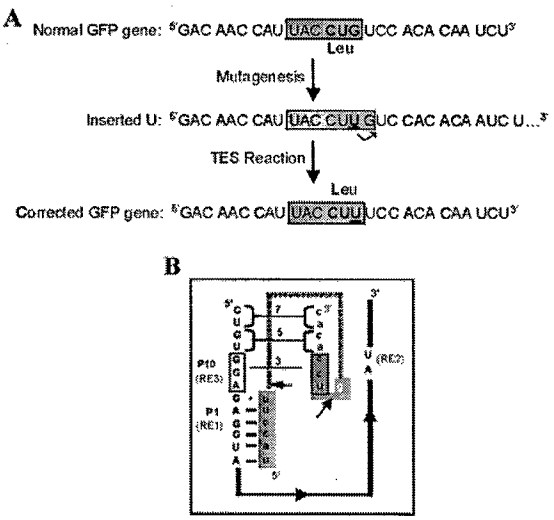
Appendix

Figure 1



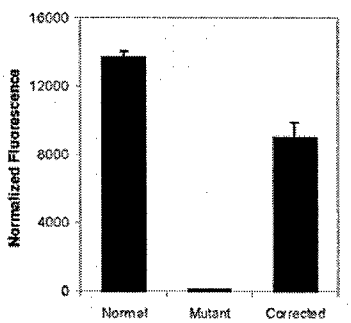
Appendix

Figure 2



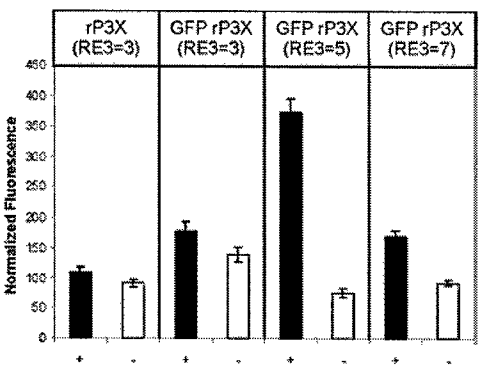
Appendix

Figure 3



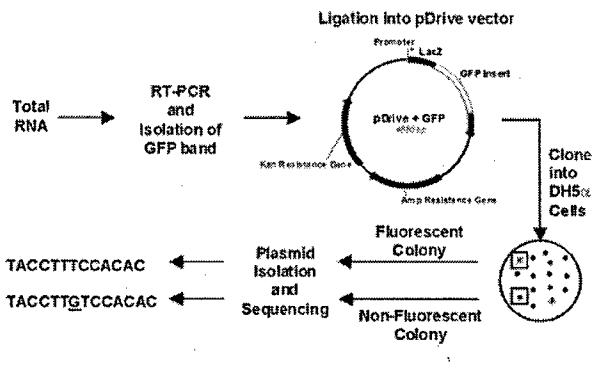
Appendix

Figure 4



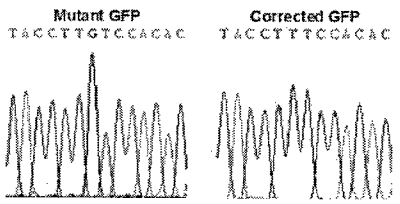
Appendix

Figure 5



Appendix

Figure 6



Appendix

Figure 7